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J.J.M. Kremers

COMPARISON BETWEEN HISTOLOGY, FUNDUSCOPY AND DENSITOMETRY FOR ASSESSMENT OF PHOTOCHEMICAL DAMAGE THRESHOLDS

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SUMMARY

In this report preliminary findings are presented on a comparison of three different techniques for assessment of photochemical damage. Aim of the experiments was to establish whether the large difference in damage threshold of two classes of photochemical damage can be attributed to damage assessment technique. In two monkeys one eye was exposed to four irradiance levels in a short (500 or 1800 s) exposure, and one eye in a long exposure (twelve hours). Total dose was about equal in both expositions. Thresholds for densitometry and funduscopy were similar to those found in more extensive experiments (Kremers and van Norren, 1989). Threshold for histological changes was about a factor two lower, in agreement with the literature (Fuller et al., 1980). Given a certain dose, long exposures showed slightly less histologic damage than short ones, indicative for a recovery process with a time constant in the order of days.

Damage was mainly present in the photoreceptor outer segments and in the RPE. The receptors were strongly vesiculated and dispersed. The RPE was often swollen, and had lost its intracellular structure.

The conclusion is that these preliminary data give no indication that method of damage assassment explains the large damage threshold differences between the two classes of photochemical damage.

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Vergelijking tussen histologie, funduscopie en densitometrie voor het vaststellen van fotochemische schadedrempels

J.J.M. Kremers

SAMENVATTING

Dit rapport behelst een verkennende vergelijking tussen drie methoden om fotochemische schade vast te stellen. De achterliggende vraag is, of het grote verschil in drempelschade tussen twee klassen fotochemische schade, het gevolg zou kunnen zijn van de schadebepalingstechniek. Bij twee apen werd telkens één oog blootgesteld aan 4 irradiantieniveaus in een korte expositie (500 of 1800 s) en één oog aan 4 niveaus in een lange expositie (12 hr). De totale dosis was bij beide expositieduren ongeveer gelijk. Drempels voor funduscopie en densitometrie bleken ongeveer gelijk te zijn aan die in eerdere experimenten (Kremers en van Norren, 1989). De drempel voor histologische schade was ongeveer een faktor twee lager dan die voor funduscopie, zoals in de literatuur eerder beschreven (Fuller et al., 1980). Gegeven een vaste dosis bleek de schade bij lange expositie iets kleiner dan bij korte expositie, hetgeen wijst op een herstelproces met een tijdsconstante in de orde van dagen.
Schade werd voornamelijk gevonden in de buitenste segmenten van de fotoreceptoren en in het pigment epitheel. De receptoren vertoonden blaasjes en waren vervormd. Het pigmentepitheel was gezwollen en vertoonde structuurverlies.
De conclusie is dat dit beperkte onderzoek geen enkele indicatie oplevert dat de methode van schadebepaling de grote verschillen in drempeldosis zou kunnen verklaren die tussen de twee schadeklassen bestaan.

1 INTRODUCTION

The main theme of the thesis of Kremers (1989) was: Can we possibly attribute the distinction between two classes of experimental data on photochemical retinal damage to differences in experimental techniques? So far, neither field width, nor anesthesia has proven to give a cue. Rapp et al. (1989) and Van Norren and Schellekens (1990) have recently added animal model as an unlikely explanation for the class distinction, by finding Ham class (earlier called class II) damage in rat. Hitherto only Noell class (class I) damage had been found in this species. In this report damage assessment technique is explored as a relevant experimental parameter.

When we used funduscopy and densitometry after 12 hour exposures, damage thresholds fitted nicely in the Ham class (Kremers and van Norren, 1989). We had expected to find a transition from Noell class to Ham class. Did we possibly miss it because of the damage assessment technique? Since in Noell class threshold damage remains limited to the photoreceptors, it is quite likely that funduscopy would reveal no damage. Perhaps subtle damage might also have escaped densitometry.

When two monkeys had to be sacrificed for other reasons, we grasped the opportunity to perform a pilot study on their retinae. We exposed one eye for 12 hours and the other eye for a much briefer period (500 or 1800 s). Long exposures were interesting because these might show the transition from Noell to Ham class depending on damage criterion, and the short exposures served as a control for undisputed Ham class damage. For Ham class damage a drop in threshold of about a factor two is expected when changing from funduscopy to histology (Fuller et al, 1980). In view of the limited nature of the present experiments quantitative assessments of thresholds had to be incomplete.

2 METHODS

Two male macaques (Macaca fascicularis) were available for histology because of overcompleteness in a monkey colony. The experimental set up was identical to that described by Kremers and van Norren (1989). Thus, in each eye four patches of 4 degrees in diameter were simultaneously exposed with different irradiances. In the first animal (#1) one eye was exposed for 1800 s, and immediately thereafter the other eye was exposed for twelve hours. In the second animal the same

procedure was followed, except that the first exposure lasted 500 s. At the termination of an exposure funduscopic damage was assessed in both eyes. Two days after exposure the animal was anesthetized and funduscopic and densitometric damage was assessed. Due to technical problems densitometry failed in animal #1. Thereafter the eyes were enucleated, organs were removed for use in other investigations and the animals were euthanized.

The eyes were fixated in in a cacodylate buffered solution of 1% paraformaldehyde and 1.25% glutaraldehyde, pHG 7.3, for at least one week. Two incisions at the ora serrata allowed for more complete and faster fixation. After fixation the eyes were cut in half, and the irradiated patches were cut out with razor blades together with a non-exposed patch which served as blank. The irradiated patches were identified with the aid of a fundus photograph on which the these patches were marked. Funduscopic damage was visible on the preparations. Thereafter, the patches were processed for light- and electron-microscopy. The preparations were coloured with toluidin blue for light microscopic preparations and with lead citrate and uranyl acetate for electron microscopy. The ultrathin sections were inspected and photographed in a Philips EM 201 transmission electron microscope. Unfortunately, two eyes (#1, 1800 s and #2, 12 hr) proved to be unsuitable for histological damage assessment because of extensive retinal detachment during the fixation process, probably caused by mechanical deformation.

3 RESULTS

Table 1 summarizes the thresholds found with the different techniques. The funduscopic and densitometric damages at different time intervals after exposure matched the damage data presented by Kremers and van Norren (1989): directly after exposure, no funduscopic lesions could be seen. Twelve hours after the 500 and 1800 s exposures, threshold funduscopic damage was found at an irradiant dose of about 360 J.cm-2. Two days after exposure the irradiant dose for threshold funduscopic damage was at about 200 J.cm-2 for both long and short exposures, very near the threshold of 230 J.cm-2 found by Kremers and van Norren (1989). Densitometric damage was only assessed in animal #2. With the criterion described by Kremers and van Norren (1989) threshold was about 200 J.cm-2, confirming the close relation between funduscopic and densitometric damage described in the same paper.

Table 1 Summary of thresholds (in J.cm-2) for light damage with funduscopy, densitometry, and histology (n.m.= not measured; det.= extensive retinal detachment).

animal	exposure time	funduscopy			densitometry	histology
		direct	12h	2 days	2 days	2 days
1	1800 s	>655	655	130-360	n.m.	det.
1	12 hr		626	125-346	n.m.	125
2	500 s	>571	360	188	188	<360
2	12 hr		>492	246	246	det.

Fig. 1 shows light- and electron-microscopic preparations of unexposed parts of the retina. Electron-microscopy mainly concentrated on photoreceptors and RPE, because most extensive damage was found in these layers. This is in accordance with observations of other investigators (e.g. Ham et al., 1978; Tso, 1972; Irvine et al., 1984; Noell et al., 1980). The slight vesiculation of the outer segments and the swelling of the inner segment mitochondria is probably due to delayed fixation, which might have been avoided had the eye been perfused.

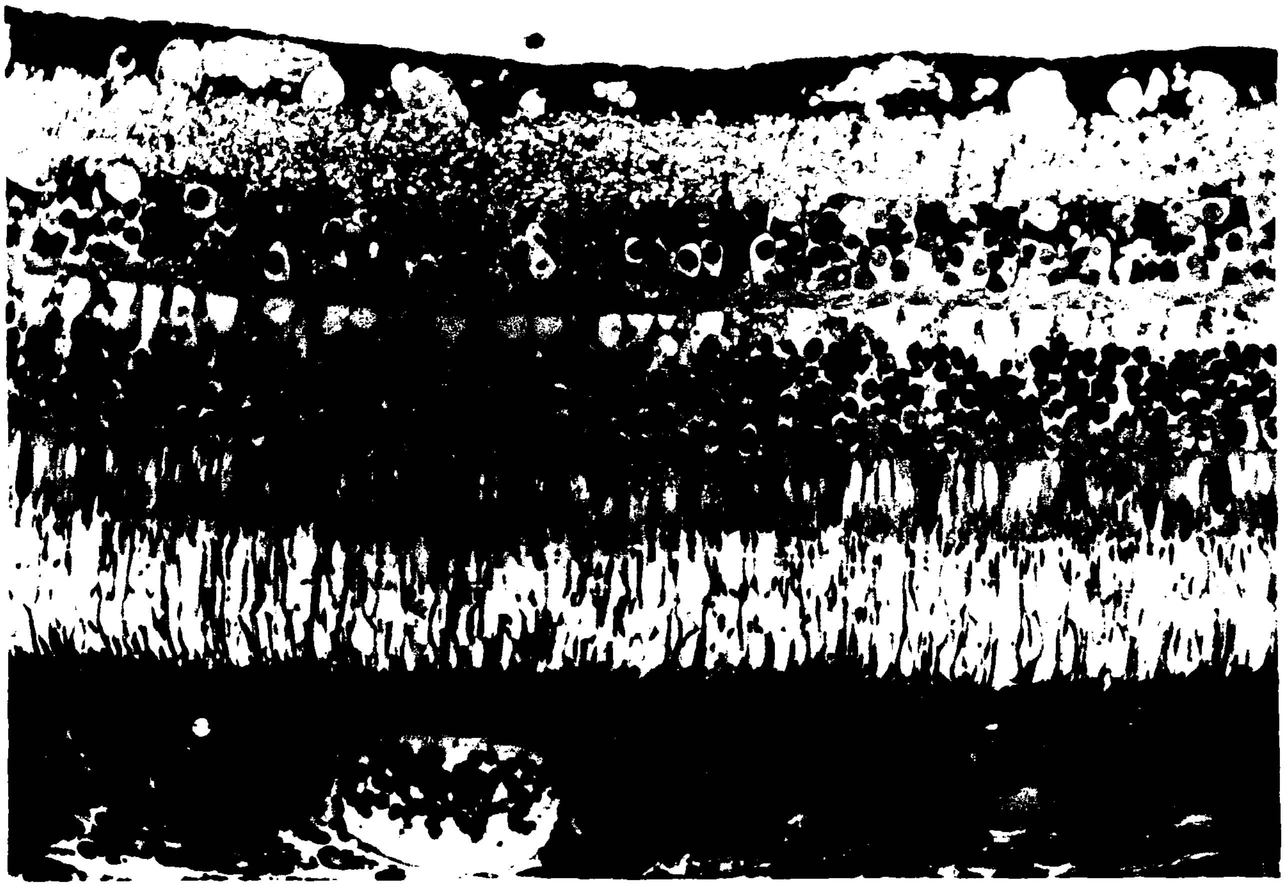

Fig. 1 Light-(a) and electronmicroscopic (b) preparation of an unexposed part of the retina. The organelles and structures are easily discernable in the RPE cells. The outer segments of the photoreceptors are long, straight and parallel. The slight vesiculation in the outer segments and the swelling of some mitochondria are probably the result of delayed fixation, and therefore an artifact.

Fig. 1b

Fig. 2 presents light- and electron-microscopic preparations of the patch exposed for 500 s to a total retinal irradiant dose of 360 J.cm-2, resulting in a funduscopic and densitometric supra-threshold damage. On the light-microscopic slides the transition from an undamaged (at the left side of the photo-micrograph) to a damaged part of the retina is clearly visible.

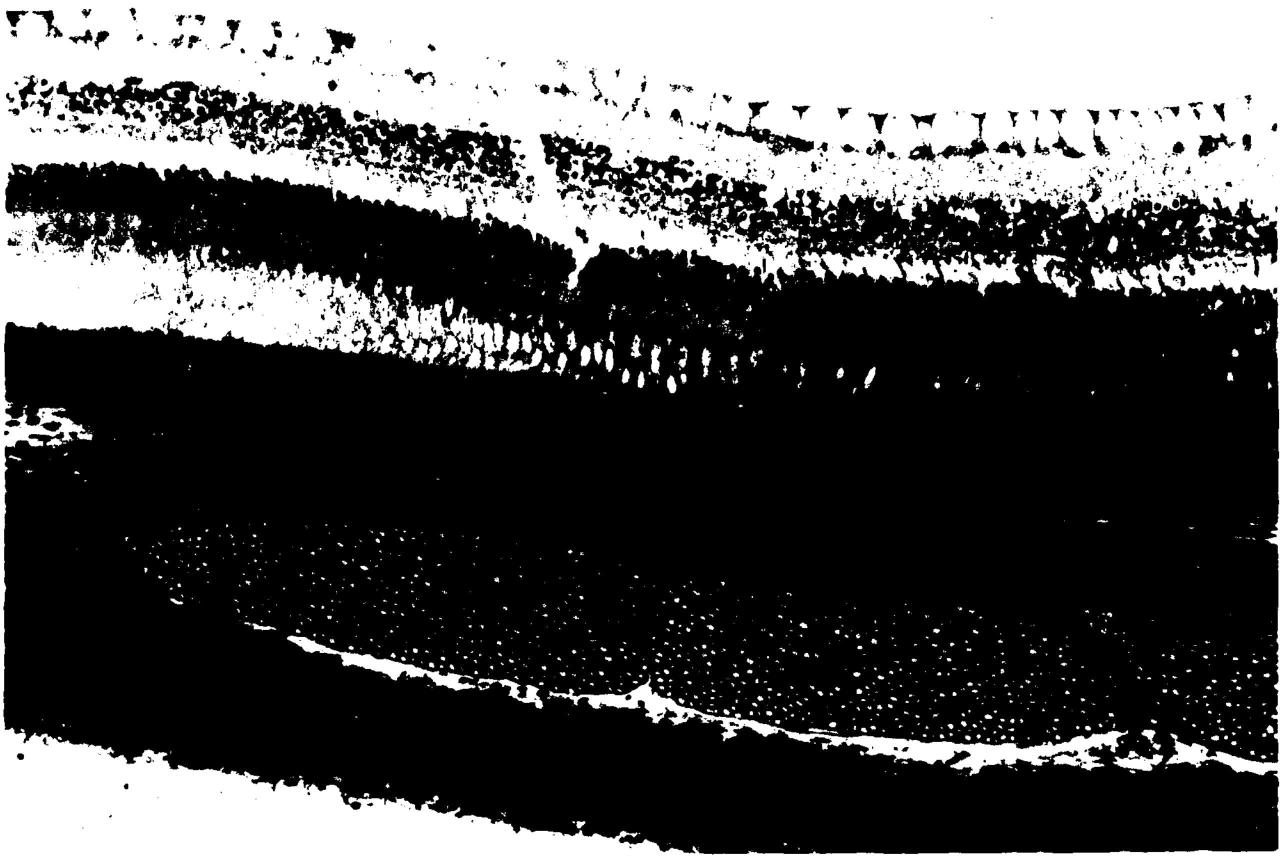

Fig. 2 The microscopic appearances of a retinal patch two days after an exposure of 500 s to a total irradiant dose of 360 J.cm-2. The light-micrograph (Fig. 2a) shows the transition area from undamaged (left) to damaged retina (right). The damaged part of the outer retina has a more dense appearance. The following features can be seen: pyknosis in the nuclei of the photoreceptors. Loss of the parallel orientation of the outer segments, resulting in a thinner but denser outer segment layer. Swellings in the RPE. The electron-micrographs (Fig. 2b) show the loss of the parallel orientation of the stacks and the vesiculations within the outer segments, and the loss of texture within the RPE cells.

Fig. 2b

Damage is confined to the outer layers: The cell nuclei of the outer nuclear layer seem to be more heavily coloured, which possibly is an indication for pyknosis. Further, extensive damage is present in the outer segments of the photoreceptors and the RPE. The outer segment layer is thinner but more dense than in the unexposed parts. The parallel orientation of the outer segments is lost. The RPE seems to be swollen, and the melanin granules, present at the apical side of the cells, are clumped and dispersed over the cells.

The electron-microscopic preparation shows the outer limiting membrane to be less tight. The inner segments are relatively intact. As observed on the light-microscopic slides, the outer segment layer is compressed. The outer segments are heavily vesiculated. In the RPE, many dense membranous structures can be seen, possibly originating from phagocytized outer segments. Microvilli with melanin granules, as seen on the unexposed patch, are not present. Many vesicles are present. The cytoplasm seems to have lost its structure, as if dissolved.

Fig. 3 shows an electron-micrograph of the damage after a 500 s exposure to an irradiant dose of 571 J.cm-2, close to a dose causing permanent damage (Kremers and van Norren, 1989). All the features of damage present in Fig. 2 are, even more pronounced, present here as well. In the RPE hardly any structures can be recognized. The rupture of Bruch's membrane is probably an artifact, as it was sometimes also observed in unexposed patches.
Uncertainty in the localization prevented analysis of the 500 s patches with doses 66 and 188 J.cm-2.

Fig. 3 Electron-(b) micrographs of a patch exposed for 500 s to a total irradiant dose of 571 J.cm-2. All features of damage as shown in Fig. 2 are, though more severely, present.

Fig. 3

In Fig. 4 microscopic preparations are shown of damages two days after 12 hour exposures with total irradiant dose of 346 J.cm-2, being a funduscopic supra-threshold damage. The same damage features as for the 360 J.cm-2, 500 s exposure were present, for both photoreceptors and RPE.

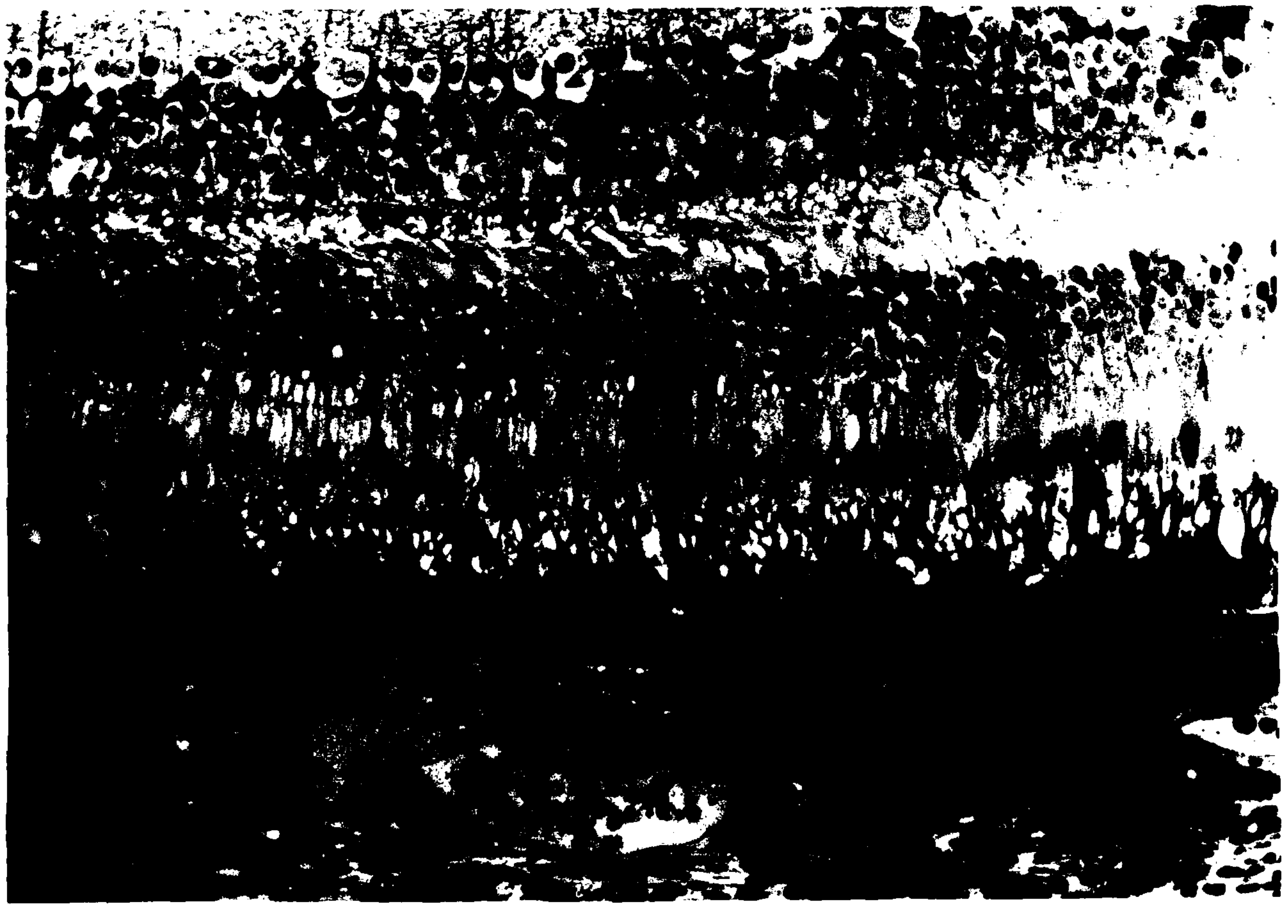

Fig. 4 Light- and electron-micrographs of a patch two days after a 12 hr exposure with total irradiant dose of 346 J.cm-2. Again the same features as in Fig. 2 are present, though less severe. In the RPE the nucleus is still present, indicating that the cell might still be viable. In the RPE cells there is some texture left, and the melanin granules are still more or less present at the apical side of the cell. Thus, though the patch shown in Fig. 2 received a similar total irradiant dose, the damage of that patch was more severe. This indicates that during twelve hour exposures repair processes may play a role.

Fig. 4b

Yet the extent of the damage is somewhat less, indicative for the effects of a repair process. It was striking to observe that retinal detachment was absent in patches with funduscopic supra-threshold damages. Thus, it seemed as if photoreceptors and RPE were "glued", like in thermal coagulation.

In Fig. 5, finally, the light-microscopic appearance after a twelve hour exposure with a funduscopic subthreshold dose of 125 J.cm-2 is presented. Alterations in the photoreceptors are observed but to a much lesser extent than at 346 J.cm-2.

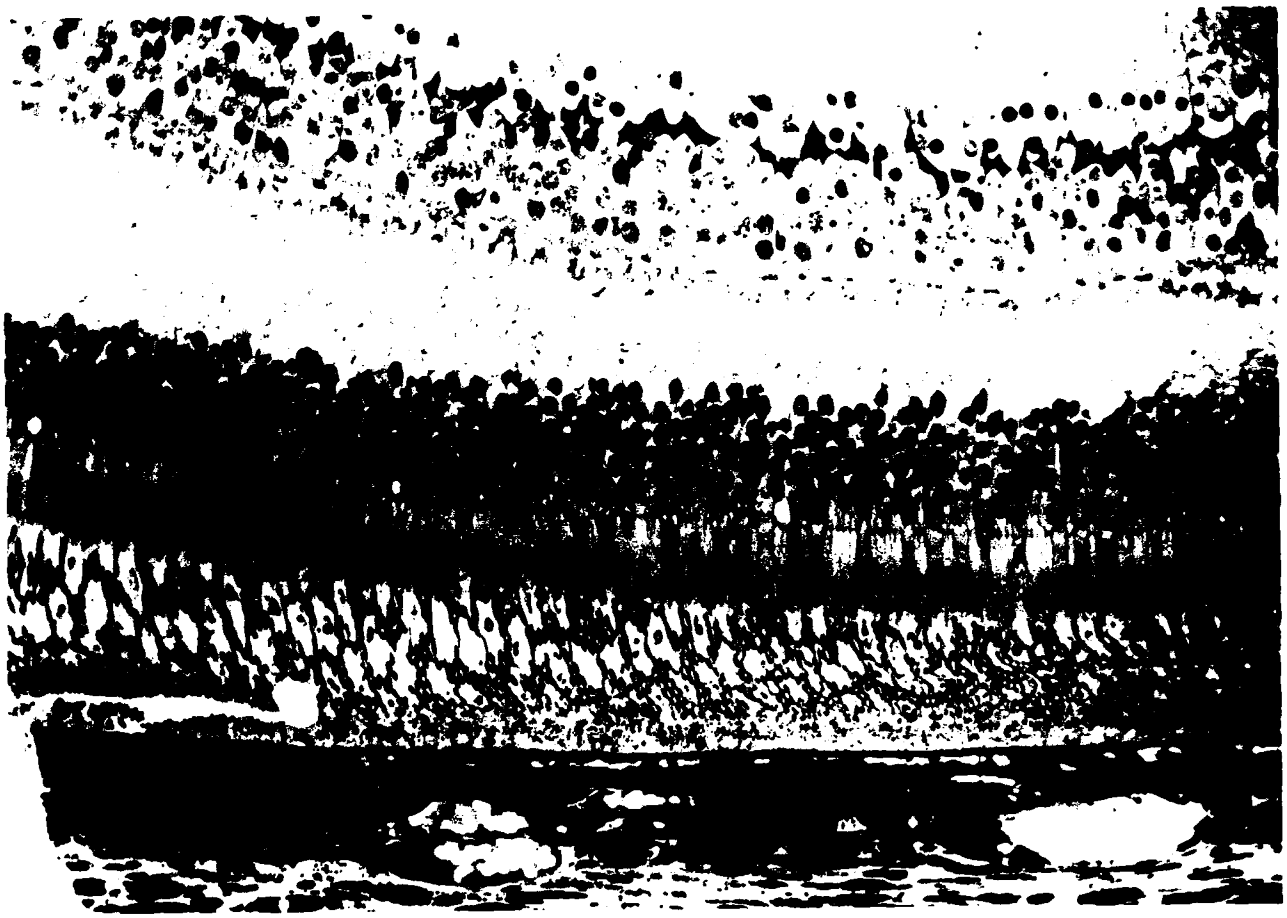

Fig. 5 Photo-micrograph of a patch two days after a 12 hr exposure with total irradiant dose of 125 J.cm-2. The transition from undamaged (left) to damaged area can be seen. The damage is much less severe as in the previous figures, indicating a near threshold damage.

The RPE does not seem to be damaged at all. We judge these changes as being close to microscopic threshold damage. No damages were visible after still lower irradiant doses. But, retinal detachment and difficulties with identifications of the exposed spots might have caused that we overlooked subtle damages, so that we cannot be definite about the consequences of these low irradiances. However, the slight changes found for the 125 J.cm-2 exposure and the previously found sharp transition from sub- to supra-threshold for funscopic and den-sitometric damages (Kremers and van Norren, 1989) make it rather improbable that damage was present for the lowest doses.

4 DISCUSSION

The threshold data with funduscopy and densitometry fully match the previously observed thresholds (e.g. Ham et al., 1980; Kremers and van Norren, 1989), both for long and short exposures. For the twelve hour exposure it was found that the microscopic threshold damages were about a factor two lower than the funduscopic thresholds. Fuller et al. (1980) had similar results with much shorter exposures (about 500 s). This is further evidence for the conclusion of Kremers and van Norren (1989) that in all these exposures only one type of damage is involved.

Microscopic damage after 12 hour exposures was somewhat less than after 500 s exposures with the same dose. This points to a recovery process with a fairly long time constant for recovery as suggested by Griess and Blankenstein (1981).

The histology partly failed because of the difficulty identify irradiated patches of only 4° (1 mm), with a separation of 1-2°. This despite the fact that all patches were carefully sketched on fundusphotos. Irradiated patches should at least be 10°.

We conclude that in the few experiments done so far, all damages were indicative of the Ham class. Possibly, even lower irradiances, and longer exposures, are required to produce Noell class damage. A way to accelerate damage production might be elevation of body temperature during exposure. As in rats, the use of green instead of white light might also be useful in monkeys to promote damage of the Noell class.

Soesterberg, October 24, 1989

Dr. D. van Norren

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15. ABSTRACT (MAXIMUM 200 WORDS, 1044 BYTE)

In this report preliminary findings are presented on a comparison of three different techniques for assessment of photochemical damage. Aim of the experiments was to establish whether the large difference in damage threshold of two classes of photochemical damage can be attributed to damage assessment technique. In two monkeys one eye was exposed to four irradiance levels in a short (500 or 1800 s) exposure, and one eye in a long exposure (twelve hours). Total dose was about equal in both expositions. Thresholds for densitometry and funduscopy were similar to those found in more extensive experiments (Kremers and van Norren, 1989). Threshold for histological changes was about a factor two lower, in agreement with the literature (Fuller et al., 1980). Given a certain dose, long exposures showed slightly less histologic damage than short ones, indicative for a recovery process with a time constant in the order of days.
Damage was mainly present in the photoreceptor outer segments and in the RPE. The receptors were strongly vesiculated and dispersed. The RPE was often swollen, and had lost its intracellular structure.
The conclusion is that these preliminary data give no indication that method of damage assessment explains the large damage threshold differences between the two classes of photochemical damage.

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